



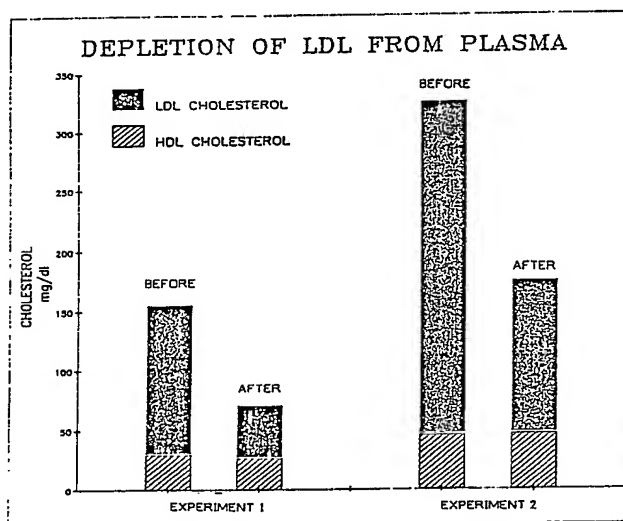
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>A01N 1/02, A61M 37/00</b> <b>B01D 15/00, G01N 33/543</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/12390</b> <b>(43) International Publication Date:</b> 28 December 1989 (28.12.89)
<b>(21) International Application Number:</b> PCT/US88/02067 <b>(22) International Filing Date:</b> 20 June 1988 (20.06.88)  <b>(71) Applicant:</b> E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).  <b>(72) Inventor:</b> WHITE, Charles, Thayer ; 4 Lombardy Drive, Wilmington, DE 19803 (US).  <b>(74) Agent:</b> WOLFSON, Herbert, M.; E.I. du Pont de Nemours and Company, Legal Department, Patent Division, Wilmington, DE 19898 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).		<b>Published</b> <i>With international search report.</i>

**(54) Title:** BIOCOMPATIBLE, SUBSTANCE-SPECIFIC REAGENTS FOR TREATING PHYSIOLOGICAL FLUIDS

**(57) Abstract**

The preparation and method of use of biocompatible water-insoluble reagents for removal of substances from physiological fluids is described. The reagent, comprising a carrier substrate with a layer of immobilized binding agent and biocompatibility agent, is produced by contacting an activated carrier substrate concomitantly or sequentially with binding agent and biocompatibility agent. The biocompatibility agent, which comprises an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm, reacts with the substrate surface through its nucleophilic moiety. The reagent may be incorporated in conventional extracorporeal systems for hemo- or plasmapheresis to remove substances such as low-density lipoprotein.



*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Fasso	HU	Hungary	NO	Norway
BG	Bulgaria	IT	Italy	RO	Romania
BJ	Benin	JP	Japan	SD	Sudan
BR	Brazil	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

TITLE  
BIOCOMPATIBLE, SUBSTANCE-SPECIFIC REAGENTS  
FOR  
TREATING PHYSIOLOGICAL FLUIDS

Technical Field

10           This invention relates to the preparation and  
use of biocompatible water-insoluble reagents for  
removal of substances from physiological fluids  
comprising specific binding ligands and  
biocompatibility agents immobilized on the surface of  
15 a carrier substrate.

Background of the Invention

          The selective removal of deleterious substances  
20 from blood, blood plasma, or other physiological  
fluids can provide important therapeutic benefits.  
For example, high levels of circulating lipids,  
particularly cholesterol, which is carried in low  
density lipoprotein (LDL), is a cause of  
25 atherosclerosis. Left unchecked, atherosclerosis can  
lead to serious circulatory system diseases resulting  
in stroke and/or myocardial infarction. Reduction in  
the plasma LDL levels is believed to significantly  
reduce the incidence of such events in high-risk  
30 patients [A.M. Gotto, et al Circulation, 69,  
1065A-1090A(1984)]. Selective removal of LDL should  
be an effective method for reducing atherosclerosis.  
Similarly, the ability to intervene in arthritis by  
removal of circulating rheumatoid factor, immune  
35 related diseases by removal of antibodies, or cancer  
by removal of malignant cells are being pursued in

2

medical science. At present, selective depletion  
plasmapheresis, which involves passage of 2-4 liters  
of blood or plasma through an extracorporeal device  
5 containing the selective adsorbent, represents an  
attractive therapy.

Clinical use of such treatment modalities, of  
necessity, brings the physiological fluid into contact  
10 with foreign surfaces. In the case of blood and blood  
plasma, one of the sequelae that can result is  
activation of the complement cascade which can lead to  
life threatening anaphylaxis. In practicing such  
treatment modalities, blood or plasma has been  
15 anticoagulated using citrate and/or heparin. Citrate  
is often employed as much for its ability to interfere  
with the complement cascade as for its ability to  
block coagulation. The reliance upon citrate to  
inhibit complement can be risky as citrate is toxic at  
20 doses very close to its dose for use as an  
anticoagulant. Typically, citrate is employed at the  
dose where mild toxicity is observed (numbness and  
tingling of the lips). The toxicity of citrate is due  
to calcium chelation interfering with normal nerve  
25 conduction. The usefulness of extracorporeal devices  
containing highly selective adsorbing materials is  
therefore dependent not only on the specificity and  
affinity of the material, but also its  
biocompatibility with body tissues and biochemical  
30 systems.

Stoffel and Demant [Proc. Nat. Acad. Sci. USA,  
78, 611-615 (1981)] describe the application and  
effectiveness of immunoadsorption chromatography to  
35 remove low density lipoprotein from plasma. Utilizing  
polyclonal anti-swine LDL antibodies covalently

3

attached to Sepharose CL-4B, swine were treated with an extracorporeal device having a plasma separator and the immunoadsorbent interposed in series in an arteriovenous shunt. Plasma LDL concentration was found to progressively decrease as the duration and flow volume increased in the treatment.

Koren et al. [Biochimica et Biophysica Acta, 876, 202-207 (1986)] disclose the use of a mouse monoclonal anti-human-LDL antibody coupled to an agarose support to remove LDL from plasma samples. No apheresis was performed on any organism.

Parker et al. [Proc. Nat. Acad. Sci. USA, 83, 777-781 (1986)] have treated patients by LDL-pheresis utilizing sheep polyclonal anti-LDL antibodies attached to sepharose. Significant reductions in LDL levels were achieved by the therapy.

20

European Patent Application 83112042.3 published June 6, 1984 discloses the use of immobilized heparin or dextran sulfate to remove LDL from plasma. The adsorbent utilized comprised a water-insoluble porous hard gel to which a binding ligand was attached. Ligands were attached through reaction with epoxy groups introduced into polymer gels by reaction with epichlorohydrin or a polyoxirane compound, or in the case of inorganic gels, with epoxy groups introduced by reaction with epoxy silane coupling agents. Gels with surface hydroxyl groups were therefore preferred. While a number of selective binding ligands were disclosed, only heparin and dextran sulfate (adsorbents removing numerous plasma proteins) were exemplified as LDL removal systems.

Japanese Patent Application JP60087854 discloses a blood purifying adsorber for removal of malignant substances or cells from blood or plasma. The  
5 adsorbent comprises a carrier such as glass, charcoal, cellulose triacetate, and various polymers to which a negatively charged compound and an organic ligand have been attached. The attachment requires the use of  
10 strong electrophilic reagents, however, which cannot be utilized in concomitant or subsequent reactions incorporating complex biological binding ligands.

European patent application EP-103184A discloses biospecific polymers carrying immobilized biological  
15 agents. The biocompatible polymer support is generally a hydrogel or other wettable polymer to which a biological agent has been covalently bounded. No biocompatibility agents are disclosed which would generally provide protection against adverse reactions  
20 in vivo.

Japanese patent application J602239425 discloses the use of solid carriers with attached monoclonal antiapolipoprotein B antibodies to remove LDL from  
25 human plasma. No biocompatibility agent was utilized to help protect against adverse reactions with tissues or systems in vivo.

Japanese patent application JP59200655 discloses  
30 the use of an adsorbent consisting of an insoluble carrier, preferably hydrophilic, with an attached organic compound containing a negatively charged group. The immobilization of the binding ligand is through the negatively charged group. The application  
35 of the adsorbent to remove malignant cells is discussed.

5

At this time there exists a clear need for an adsorbent reagent which incorporates specific binding ligands and biocompatibility agents to remove pernicious substances from physiological fluids. Such reagents should exhibit both high selectivity and protection against activation of the complement system or blood coagulation. Enhanced biocompatibility of the reagent would enable a reduction in the amounts of anticoagulants administered. The activity of complex biological ligands is highly sensitive to the immobilization technique. A method of preparing such reagents is also required which is mild enough to avoid denaturation, yet flexible enough to permit concomitant or sequential immobilization of binding ligands and biocompatibility agents.

20

25

30

35

Summary of the Invention

A process for immobilizing both specific binding  
5 ligands and biocompatibility agents on a wide variety  
of carrier substrates has been discovered. The novel  
reagents produced remove substances from physiological  
fluids while minimizing adverse reactions such as  
complement activation. Specifically, one aspect of  
10 this invention is a water-insoluble reagent for  
removal of a substance from physiological fluids  
comprising (a) a carrier substrate, and immobilized on  
the surface of said carrier substrate, (b) a binding  
ligand specific for said substance, and (c) a  
15 biocompatibility agent comprising an organic molecule  
with a nucleophilic moiety and a negatively-charged  
moiety separated by a spacer arm, said  
biocompatibility agent immobilized on said surface  
through reaction with said nucleophilic moiety.  
20 Another aspect of this invention involves a process  
for preparing such a water-insoluble reagent for  
removal of a substance from physiological fluids  
comprising the step of contacting and reacting a  
carrier substrate with an aqueous solution of a  
25 binding ligand specific for said substance and  
biocompatibility agent comprising an organic molecule  
with a nucleophilic moiety and a negatively-charged  
moiety separated by a spacer arm. This process can  
comprise both simultaneous or sequential contacting  
30 and reacting of the carrier substrate with the aqueous  
solutions of binding ligand and biocompatibility  
agent. Yet another aspect of the invention involves a  
method of treating a physiological fluid to remove a  
substance comprising contacting said fluid with a  
35 water-insoluble reagent as described above. As a  
final aspect, the instant invention provides an



7

apparatus for the extracorporeal treatment of physiological fluids to remove a specific substance comprising means for withdrawing whole blood from a mammal, means for separating plasma from the whole blood, means for treating said plasma including a chamber containing a water-insoluble reagent as described above which will interact with and deplete said substance from the plasma, and means for recombining the substantially substance depleted plasma with the remainder of the whole blood and for returning the recombined whole blood to the mammal.

In the case of LDL removal from blood plasma, preferred reagent and processes would utilize an activated neutral polymer carrier substrate, polyclonal anti-LDL antibody, and a biocompatibility agent with immobilization carried out in two steps at a pH of from about 4 to about 10 controlled by a non-nucleophilic buffer. Most preferred, by virtue of convenience of preparation and/or efficiency of operation is the reagent comprising the immobilization of monoclonal anti-LDL antibody and sulfamic acid on tresyl-sepharose in a one-step concomitant reaction at about pH 8 in potassium phosphate buffer.

30

35

### Brief Description of the Drawings

Figure 1 is a bar graph representing LDL and HDL  
5 plasma concentrations pre- and post-treatment for two  
experiments utilizing human plasma in vitro.

### Detailed Description of the Invention

#### Preparation of Materials

10

This invention provides a versatile method for  
synthesizing novel biocompatible reagents for removal  
of specific substances from physiological fluids  
through the immobilization of specific binding ligands  
15 and biocompatibility agents on substrate carriers  
under mild conditions which preserve the activity of  
sensitive biological ligands. In the context of this  
disclosure, a number of terms shall be utilized. As  
used herein, "carrier substrate" is a solid supporting  
20 matrix "activated" by well-known coupling chemistries  
to introduce reactive surface moieties susceptible to  
nucleophilic attack and displacement by binding  
ligands or biocompatibility agents to be immobilized  
on the carrier substrate. "Binding ligand" refers to  
25 any substance, or group of substances, which has  
specific binding affinity for the substance to be  
removed from the physiological fluid to the exclusion  
of other substances. The binding ligand may consist  
of a polyclonal or monoclonal antibody in the form of  
30 whole antiserum or ascites fluid, an IgG fraction or  
as active fragment of such materials. Other specific  
binding proteins, for example lectins, antigens, or  
protein A, may be utilized. As used herein,  
"biocompatibility moiety" refers to a substance which  
35 renders the carrier substrate more biocompatible  
vis-a-vis complement activation. The term

"nucleophilic moiety" refers to chemical groups, for example  $\text{NH}_2$ ,  $\text{SH}$  or  $\text{OH}$ , which carry an unshared pair of electrons and generally attack another molecule at a site where the atomic nucleus is poorly shielded by outer electrons. As used herein, the term "negatively charged moiety" refers to chemical groups, for example  $\text{OSO}_3^-$ ,  $\text{RSO}_3^-$ ,  $\text{RSO}_2^-$ ,  $\text{COO}^-$ , which carry a net negative formal charge. The nucleophilic moiety and negatively charged moiety are contained within the same molecular entity which constitutes a "spacer arm" separating the two functional moieties.

As used herein, the term "neutral polymer" refers to a water insoluble material, either natural or synthetic, that consists of repeating subunits and can be formed into a variety of possible configurations such as flat sheets, fibers, beads, porous beads, membranes, foams, etc.

"Non-nucleophilic buffer" refers to any water buffering substance that does not contain a nucleophilic moiety such as  $\text{R-OH}$ ,  $\text{R-SH}$ ,  $\text{R-NH}_2$ ,  $\text{R}_2\text{NH}$ , and the like. "Polyclonal antibody" refers to any collection of antibodies directed against a substance where the collection of antibodies directed against a substance arise from many different antibody producing cells. "Monoclonal antibody" refers to any antibody that comes from a single antibody producing cell line.

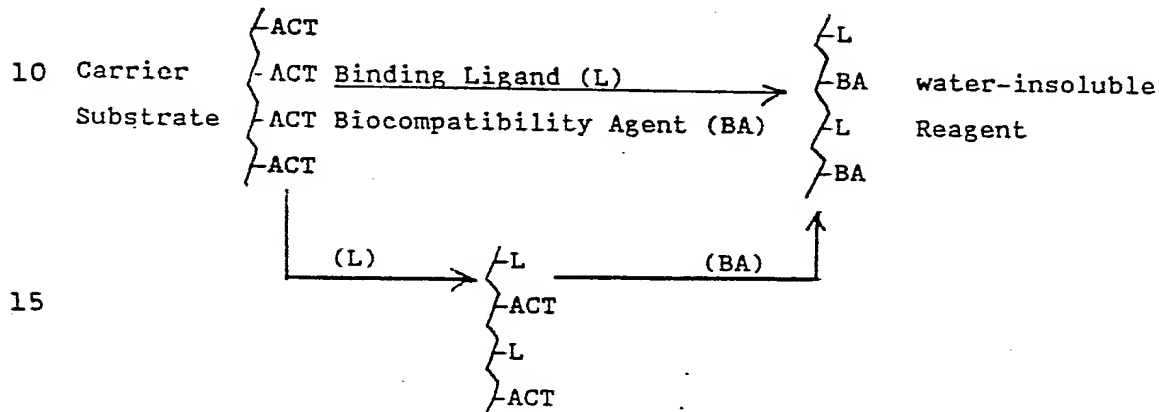
30

#### Carrier Substrate

The water-insoluble reagents of this invention involve the immobilization of binding ligands and biocompatibility agents on activated solid supporting matrices (carrier substrates) utilizing such mild

10

coupling chemistry that sensitive biological ligands  
and biocompatibility agents can be reacted  
concomitantly. The immobilization process is  
5 schematically represented in Equation 1 indicating

Equation 1

the possibility of either sequential or simultaneous  
20 reaction of the binding ligand (L) and biocompatibility  
agent (BA). Activation of various solid supporting  
matrices is achieved using chemistry appropriate for  
the material to introduce a chemical moiety (ACT)  
susceptible to nucleophilic attack by chemical groups  
25 within the molecules of the binding ligand and the  
biocompatibility agent.

A variety of materials are suitable as solid  
supporting materials for the present invention. These  
30 materials generally possess the following  
characteristics: (1) sufficient surface area to  
effectively trap and remove the target substance due  
to either the existence of pores or availability in a  
finely divided state, (2) allow for the flow of  
35 physiological fluid over or through the material when  
constrained in an extracorporeal treatment

11

apparatus, (3) surface chemical groups which can be activated for subsequent reaction with binding ligand and biocompatibility agent, and (4) stable to biological milieu. Materials fulfilling these criteria include synthetic polymers, natural polymers, silica, alumina and zirconia. The physical geometry of the solid supporting matrix can be as a particle, bead, fiber, hollow fiber, thin sheet, foams, or any other permitting fluid flow over or through the material. Table I lists a variety of commercially available carrier substrates which can be utilized to practice the instant invention. The list is representative since other materials and activations chemistries meeting the above four criteria will be known to those skilled in the art.

20

25

30

35

12

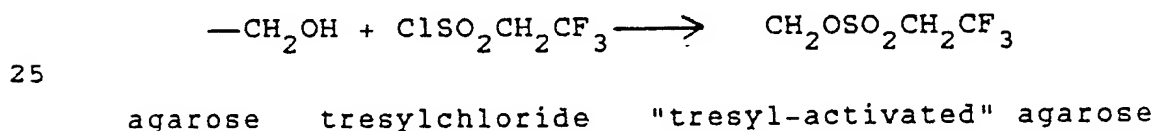
TABLE ICARRIER SUBSTRATES

5	<u>Carrier Substrate</u>
	Affigel 10 (NHS activated agarose, BioRad Co, Richmond CA)
	Affigel 15 (NHS activated agarose, BioRad Co, Richmond CA)
	Tresyl-Sepharose (tresyl activated agarose, Pharmacia
10	Chemical, Upsala, Sweden)
	CNBr-Sepharose (CNBr activated agarose, Pharmacia
	Chemical, Upsala, Sweden)
	Reacti-Gel (CDI activated agarose/dextran Pierce Chemical)
	Eupergit C (allylglycidyl ether containing terpolymer, Rohn
15	Pharma, Darmstadt Germany)
	Epoxy-activated agarose
	Epoxy-activated silica
	Carboxy Sepharose
	Thiol(activated) Sepharose
20	Trityl-agarose
	Oxirane acrylic beads
	p-Nitrophenol agarose
	Tosyl-agarose
	Periodate-treated agarose
25	Activated CM-cellulose
	Activated cellulose
	poly-glycidyl methacrylate
	PEI-glutaraldehyde activated nylon
	PEI-glutaraldehyde activated silica
30	Glutaraldehyde-activated agarose
	Isocyanate-activated agarose
	Maleimide-activated agarose
	Chloromethylpolystyrene

35

Activation of supporting matrix materials to produce suitable carrier substrates with surface groups susceptible to nucleophilic attack is carried out using well-known chemical techniques and process reagents. See Scouten, W.H., Affinity Chromatography: Bioselective Adsorption on Inert Matrices, Wiley & Sons, New York, (1981), herein incorporated by reference. In the case of synthetic and natural polymers, activation reagents may include cyanogen halides, epichlorohydrins, polyoxiranes, periodates, polyethyleneimines, or glutaraldehyde. In the case of the most preferred reagent for LDL removal from blood plasma, Sepharose®4B (Pharmacia Fine Chemicals, Uppsala, Sweden; beaded agarose gel) possessing surface hydroxyl groups is reacted with tresyl chloride (2,2,2-trifluoroethanesulfonyl chloride) to produce a surface sulfonate ester (Tresyl-activated Sepharose®4B, Pharmacia Fine Chemicals) as depicted schematically in Equation 2.

#### Equation 2



Such sulfonate esters have been reported to undergo nucleophilic displacement by amines, thiols, hydroxyl, and imidazole groups to produce stable chemical bonds which immobilize various binding ligands [Nilsson, K. and Mosbach, K., Biochem Biophys. Res. Comm., 102, 449-457 (1981) and Methods in Enzymology, 104, 56-69 (1984)].

35

In the case of an inorganic supporting matrix material including silica, alumina or zirconia, activation may be achieved by reaction with an

14

epoxysilane such as gamma-glycidoxypropyltrimethoxy-silane, an aminosilane such as gamma-aminopropyl-triethoxysilane, or polyethyleneimine-glutaraldehyde.

5

#### Binding Ligand

A wide variety of binding ligands may be utilized with the instant invention depending upon the specific substance to be removed from the physiological fluid. Binding ligands possessing great specificity permit the selective removal of the substance with the least overall impact on the composition of the physiological fluid being treated.

15 A representative list of binding ligands, conjugate substance to be removed, and the disease/disorder to be treated is provided in Table II.

20

25

30

35



5

TABLE II  
BINDING LIGANDS

	<u>Binding Ligand</u>	<u>Conjugate Substance</u>	<u>Disease/Disorder</u>
10	Any antigen such as Factor VIII or IX	Corresponding antibodies	Factor resistant Hemophilia
	HLA antigen	Corresponding antibodies	Transplant rejection
	DNA, RNA, nucleotides	Corresponding antibodies	SLE, or other ANA related disease e.g. Sjogren's, scleroderma, etc
	Acetylcholine receptor	Corresponding antibodies	Myasthenia Gravis
	Blood group antigen, e.g. A,B,O	Corresponding antibodies	Transplantation
15	Tumor antigens	Blocking Factors	Cancer
	Xenotypic antigens	Corresponding antibodies	Remove antibodies that neutralize antibody based therapeutics diagnostics
	Nervous tissue antigens	Corresponding antibodies	Guillan-Barre Syndrome, MS, etc
	Glomerular Basement membrane	Corresponding antibodies	Goodpasture's disease
	Anti-LDL antibodies	LDL	Hypercholesterolemia, Other lipid disorders, i.e. Fabry disease
20	Anti Idiotypic Antibodies	Other antibodies	Autoimmune disease, hyper- globulinemia, transplant rejection, any deleterious antibodies
	Protein A, protein G, or Ig binding peptide fragments	Immunoglobulins	Autoimmune disease, hyper- globulinemia, transplant rejection, any deleterious antibodies
	Anti-endotoxin antibodies	Endotoxin	Septic shock
	Anti-drug or toxin antibodies	drugs, toxins, psycho- active cpds.	Drug overdose, poisoning, psychotic syndromes
	Anti-B, or t-Cell antibodies	B, or T cells	Lymphoma or leukemia, bone marrow transplant
30	Clq, or other complement component	Immune complexes	Autoimmune diseases
	Anti-killer cell antibodies, or cell specific ligands eg IL-2	Functional white cells	Adoptive immunotherapy

35

16

It is to be understood that the binding ligands of the present invention are not limited to those set forth in Table II which are meant to be illustrative. These  
5 binding ligands may be used alone or in mixtures if appropriate.

In the case of LDL removal from blood plasma, highly specific monoclonal antibodies are most  
10 preferred as binding ligands. An anti-LDL antibody was prepared using a standard monoclonal antibody immunization and fusion protocol [Vernon T. Oi and Leonard A. Herzenberg. Selected Methods in Cellular Immunology, Eds. B. Mishell and S. Shiigi, 1980,  
15 pp 351-372] Balb/CJ mice were immunized with purified human LDL with and without complete Freund's adjuvant. The mice were boosted at regular intervals and bled at various time points for several weeks following the immunizations. Serum samples were  
20 tested in an ELISA to determine the amount of anti-LDL antibodies present in the immunized mice versus the non-immunized Balb/CJ.

Several months following the initial  
25 immunization, the mice were boosted with antigen and the first fusion was performed. A spleen cell suspension from a primed mouse was fused with SP2/O myeloma cells, mixed with an irradiated feeder spleen cell suspension, and plated out. The clones were fed  
30 at various intervals and observed for growth.

Clones were selected and assayed to determine the amount of IgG being produced, and each clone was isotyped. Clones were tested regularly to monitor IgG  
35 production. At a later date, cell supernates from selected clones were tested in a LDL ELISA to

17

determine specific activity of each selected clone. The most productive clones were selected and frozen in liquid nitrogen. Several of the better clones were  
5 subcloned and assayed to ensure that a monoclonal anti-LDL was being produced and to try and increase IgG production and LDL specificity. All of the clones producing specific anti-LDL monoclonal antibody were further assayed for other factors, such as stability,  
10 antibody production, affinity, etc. that would select the best clones for use in this invention.

#### Biocompatibility Agent

15 A number of bifunctional chemical entities incorporating both a nucleophilic moiety, for reaction with the carrier substrate, and a negatively charged moiety separated by a spacer arm are contemplated as biocompatibility agents of the instant invention. The  
20 negatively charged moiety is tethered at the surface following reaction of the nucleophilic moiety with the carrier substrate to immobilize the biocompatibility agent.

25 Many combinations of nucleophilic moiety and negatively charged moiety are contemplated by the present invention. Nucleophilic moieties including for example  $\text{RNH}_2$ ,  $\text{RSH}$ ,  $\text{ROH}$ , or  $\text{R}_2\text{NH}$  can be utilized. Negatively charged moieties including  
30  $\text{OSO}_3^-$ ,  $\text{RSO}_3^-$ ,  $\text{RSO}_2^-$ ,  $\text{CO}_2^-$ , are suitable. A representative list of biocompatibility agents is provided in Table III.

18

TABLE IIIBIOCOMPATIBILITY AGENTS

5	<u>Nucleophilic Moiety</u>	<u>Negatively Charged Moiety</u>	<u>Specific Examples</u>
	Amino	Sulfate	Sulfamic Acid Taurine Sulfanilic Acid
10	Amino	Carboxylate	All amino acids e.g. glycine, aspartic acid, cysteine
	Hydroxy	Sulfate	Isethionic Acid Hydroxybenzenesulfonic Acid
15			Glucose sulfate
	Hydroxy	Carboxylate	Lactic Acid Uronic Acids, e.g. Glucuronic Acid Glyconic Acids, e.g. Gluconic Acid
20	Thiol	Sulfate	2-Mercaptoethanesul- fonic Acid
	Thiol	Carboxylate	Mercaptoacetic Acid Thiosalicylic Acid Thiolactic Acid
25			
30			
35			

It is apparent from Table III that a number of bifunctional molecules with separated nucleophilic and negatively charged moieties can function as biocompatibility agents. It will be appreciated that the molecular structure into which such moieties are incorporated serves as a spacer arm to separate the moieties and permit independent function, i.e. reaction with carrier substrate and protection against adverse biological interactions. As such the spacer arm is not critical and one skilled in the art can select various molecular structures as spacer arms into which to incorporate the functional moieties.

#### Water-Insoluble Reagent

Binding ligands and biocompatibility agents are immobilized on the surface of the carrier substrate by nucleophilic displacement reactions involving a nucleophile of the binding ligand or biocompatibility agent and the activated moiety on the carrier substrate. In the case of protein binding ligands such as antibodies, the numerous amino groups within the antibody serve as nucleophiles for convenient reaction. The processes of the instant invention are schematically represented by Equation 1.

Simultaneous attachment of a Binding ligand and Biocompatibility Agent to the Carrier Substrate: In this process, the water-soluble reagent is prepared by adding the carrier substrate to a reaction solution containing the biocompatibility agent and binding ligand or vice versa. The carrier substrate, such as those listed in Table I, can be added as a dry material, or wetted by a suitable buffer. The reaction solution contains binding ligand at a

20

concentration of from about 10 mg/ml to about 100 mg/ml and biocompatibility agent at a concentration of from about 0.01 M to about 5 M. The reaction solution can also be buffered with any non-nucleophilic buffer, such as phosphate, at a buffer concentration of from about 0.01 M to about 5 M. The pH range of the reaction solution can vary from about 4 to about 10. After combining the reaction solution with the carrier substrate, the resultant mixture is agitated for 30 minutes or longer, up to several days if required, at any temperature that does not freeze the mixture or deform the carrier substrate, typically 4 C to 45 C. After coupling, the reaction solution is separated from the water-soluble reagent and rinsed to remove unreacted biocompatibility agent and binding ligand. The resultant derivatized carrier substrate comprises the water-insoluble reagent.

Stepwise attachment of a Binding Ligand and Biocompatibility Agent to the Carrier Substrate: In this process, the water-insoluble reagent is prepared by first reacting the carrier substrate with binding ligand and then adding biocompatibility agent to the reaction solution in a second step. This is the preferred method in cases where the binding ligand and biocompatibility agent are not compatible in the same solution, or if the reaction conditions for immobilization of binding ligand and biocompatibility agent are sufficiently different. The ranges for the reaction parameters in stepwise reactions are the same as for the simultaneous reactions described above.

#### EXAMPLE 1

Anti-LDL Water-Insoluble Reagent preparation using Simultaneous Attachment of Anti-LDL Monoclonal Antibody CLL1 (binding ligand) and Sulfamic Acid (biocompatibility agent): Tresyl-Sepharose (carrier

21

substrate; 35 gm) was sterilized by washing with acetone and filtering through a sterile sintered glass funnel. The acetone washed bead was then transferred  
5 to a sterile roller bottle (to aid transfer it is recommended that the bead be slightly wetted with acetone). To the roller bottle was added 105 ml of the reaction solution containing monoclonal antibody CLL1 (3.0mg/mL), sulfamic acid (0.25 M), and potassium  
10 phosphate (0.25 M) at pH 8.0. The slurry was gently agitated overnight (16 hr) at ambient temperature. After overnight agitation, the slurry was filtered using a sintered glass funnel and the combined sorbent washed with 1 L of citric acid (pH 3.0), followed by  
15 1 L of buffered saline (pH 7.1). The filtrate was collected for reprocessing and reuse. The sterile anti-LDL sorbent was transferred to a sterile container where merthiolate was added (final concentration 0.1%) as a preservative of sterility  
20 until further use.

## EXAMPLE 2

Anti-LDL Water-Insoluble Reagent preparation using Stepwise Attachment of Anti-LDL Monoclonal  
25 Antibody CLL1 (binding ligand) and Sulfamic Acid (biocompatibility agent): Tresyl-Sepharose (carrier substrate; 0.5 gm) was sterilized by washing with acetone and filtering through a sterile sintered glass funnel. The acetone washed bead was then transferred  
30 to a sterile centrifuge tube (to aid transfer it is recommended that the bead be slightly wetted with acetone). To the centrifuge tube was added 5 mL of the reaction solution containing monoclonal antibody CLL1 (3.0 mg/mL), and sodium bicarbonate (0.10 M) at  
35 pH 8.0. The slurry was gently agitated for 4 hours at ambient temperature. The antibody solution was

22

separated from the carrier substrate by centrifugation at 1000 x g for 10 min and decanting the unreacted antibody. Sulfamic acid (0.20 M) in sodium carbonate  
5 buffer (0.050 M), pH 9.0, was then added and the mixture agitated overnight (16 hr) at ambient temperature. After overnight agitation, the slurry was filtered using a sintered glass funnel and the anti-LDL water-insoluble reagent washed with 1 L of  
10 citric acid (pH 3.0), followed by 1 L of buffered saline (pH 7.1). The decanted supernatant and filtrate was collected for reprocessing and reuse. The sterile anti-LDL water-insoluble reagent slurry was transferred to a sterile container where  
15 merthiolate was added (final concentration 0.1%) as a preservative of sterility until further use.

#### Utility

20 The water-insoluble reagent described above can be used to selectively remove substances from physiological fluids which are bound by the binding ligand. The water-insoluble reagent would be confined in a container housing that would allow the flow of  
25 the physiological fluid through the water-insoluble reagent. If the physiological fluid were blood or blood plasma, the water-insoluble reagent would be used to remove from the blood or plasma a substance that is associated with a disease or disease process.  
30 This would be the case in a number of diseases where it is known or suspected that the removal of the disease associated substance would be beneficial. These include auto-immune diseases, metabolic disorders, and others. A more complete list of the  
35 diseases that could be amenable to this therapy, the substance(s) to be removed, and the possible ligands



23

that could accomplish this are shown in Table II. The water-insoluble reagent listed in Table I, one or more of the binding ligands listed in Table II, and one or  
5 more of the biocompatibility agents listed in Table III.

To illustrate the use of the invention for a therapeutic purpose, a volume or amount of a reagent  
10 as described above would be contained in a housing that would allow for the flow of blood or plasma. Anticoagulated blood or plasma from a patient would be pumped through the housing containing the water-insoluble reagent and then returned to the  
15 patient. The blood or plasma treated in this way would thus be unaffected except for the depletion of the desired substance bound by the binding ligand in the water-insoluble reagent. There are two parameters by which the performance or utility of the system can  
20 be evaluated: 1) the effectiveness of the binding ligand, and; 2) the effectiveness of the biocompatibility agent.

The effectiveness of the binding ligand can be  
25 determined by measuring the amount of a substance in the physiological fluid before and after passage over the water-insoluble reagent. The amount of this substance depleted is a measure of the performance of the reagent. The assay system for determining the  
30 amount of the substance removed is, of course, dependent upon the substance. To further illustrate how this would be accomplished, in the case of LDL, plasma that has been anticoagulated would be perfused over an anti-LDL water-insoluble reagent. Samples of  
35 the plasma, before and after perfusion over the combined sorbent, would be collected and assayed. Plasma samples would then assayed for total

24

cholesterol and HDL. The LDL levels are easily  
calculated from the HDL and total cholesterol values  
as the total cholesterol level of plasma is presumed  
5 to be the sum of HDL and LDL.

The total cholesterol determinations would be  
performed by standard clinical laboratory procedures.  
This procedure is a quantitative enzymatic reaction  
10 using cholesterol oxidase following chemical  
saponification of the cholesterol esters. The  
by-product of the cholesterol oxidase ( $H_2O_2$ ) is  
coupled with peroxidase and a colorimetric substrate.  
This reaction leads to a colored end product which can  
15 be measured using a spectrophotometer [Stein, E. A.,  
In, Textbook of Clinical Chemistry, Tietz, N. W., Ed.,  
WB Saunders, Philadelphia, 1986, pp 879-886, 1818, and  
1829].

20 The HDL determinations are performed by first  
precipitating the LDL, by use of a precipitating  
reagent, and assaying the supernate via the above  
total cholesterol procedure.

25 The effectiveness of the biocompatibility agent  
can be determined by measuring the amount of  
complement activation in the plasma as a result of  
exposure to the combined sorbent. Attenuation of the  
complement activation as compared to the untreated  
30 polymer carrier is the demonstration of performance of  
the biocompatibility agent. Activation of the  
complement cascade by a foreign substance occurs via  
activation of the alternate pathway. The components  
of the complement cascade that are quantitated to  
35 determine the amount of activation are  $C_{3a}$  and  
 $C_{5a}$ .  $C_{3a}$  (or  $C_{5a}$ ) is determined by a known RIA

25

technique [see Chenoweth, D.E. and Hugli, T.E.,  
J. Biol. Chem 250, 8293(1975), herein incorporated by  
reference] wherein antibodies specific to human C<sub>3a</sub>  
5 (or C<sub>5a</sub>) are allowed to react with a heparin  
anticoagulated plasma sample that has been exposed to  
the water-insoluble reagent. Use of other  
anticoagulations such as citrate, can significantly  
alter complement activation. The degree to which the  
10 specific antibody is bound to C<sub>3a</sub> (or C<sub>5a</sub>) is  
determined by a radioisotope probe. The amount of  
C<sub>3a</sub> (or C<sub>5a</sub>) in a plasma sample can then be  
calculated from the measured amount of radioactivity.  
The amounts of C<sub>3a</sub> and C<sub>5a</sub> are summed to give a  
15 value representative of the total invitro complement  
activation of the tested material.

The water-insoluble reagent can be contained in  
various shaped housings depending upon the physical  
20 form of the solid support material or the  
application. The shape of the apparatus employed is  
not critical to this disclosure, any apparatus that  
restrains the water-insoluble reagent and allows for  
flow of the physiological fluid can be used.

25

Utilizing the above techniques, the effectiveness  
of the water-insoluble reagent to remove LDL from  
plasma can be demonstrated by measuring the LDL levels  
before and after perfusion of a plasma sample over the  
30 water-insoluble reagent. Employing the water-insoluble  
reagent of Example 1 comprising anti-LDL monoclonal  
antibody CLL1 (binding ligand) and sulfamic acid  
(biocompatibility agent) immobilized on tresyl  
Sephacrose, demonstration of LDL binding to the  
35 immunosorbent and depletion of LDL from plasma is  
illustrated by the two experiments shown in Figure 1.

26

The values for LDL cholesterol (solid bars) and HDL cholesterol (hatched bars; total cholesterol is the sum of HDL and LDL) before and after plasma treatment by the immunosorbent are presented. The immunosorbent effectively depletes LDL from plasma whether a high cholesterol (>300 mg/dl) or a low cholesterol (155 mg/dl) plasma is used. Also note that HDL levels are unaffected by the treatment with the LDL immunosorbent, attesting to the specificity of the water-insoluble reagents.

The effectiveness of the biocompatibility agent to attenuate complement activation in the plasma as compared to the untreated polymer carrier is shown in Table IV.

Table IV

<u>Material</u>	<u>C<sub>3a</sub></u> <u>µg/ml</u>	<u>C<sub>5a</sub></u> <u>µg/ml</u>	<u>Total</u> <u>Activation</u>
Untreated Sepharose	82	19	101
Hydrolyzed tresyl-Sepharose	56	15	71
Tris-Sepharose	60	3	63
Sulfanilic-Sepharose	44	4	48
Sulfamic-Sepharose	43	4	47

As mentioned previously, total complement activation is the sum of C<sub>3a</sub> and C<sub>5a</sub> and is expressed as µg/ml in the test plasma. Sulfanilic and sulfamic treatment result in the use of a biocompatibility agent from Table III and a significant reduction in the complement activation of that material is seen. Treatment with Tris (tris(hydroxymethyl)aminomethane, Sigma Chemical Company) or water hydrolyzed

27

tresyl-Sepharose does not incorporate the negatively charged moiety onto the surface and does not confer the protection obtained from the use of the  
5 biocompatibility agent.

A combination sorbent prepared with a binding ligand and a biocompatibility agent confers both binding and biocompatibility properties to the  
10 water-insoluble reagent. Specifically, the water-insoluble reagent prepared using the anti-LDL antibody CLL1 and sulfamic acid (Example 1) demonstrate the combined effect; the ability of the biocompatibility agent to attenuate complement, and  
15 the ability of the binding ligand to bind and remove LDL. The results are shown in Table V which lists the amount of LDL bound in mg per ml of water-insoluble reagent as well as the associated complement activation.

20

25

30

35

28

TABLE V

REAGENT CHARACTERISTICS

5			
	<u>Material</u>	<u>Amount LDL Bound, mg/ml</u>	<u>Complement Activation</u>
	Untreated Sephadex	0	143
10	Sephadex-CLL1-Tris	4.0	72
	Sephadex-CLL1- Sulfamic Acid	4.0	53
	Blank	N/A	5

15

EXAMPLE 3

20     Use of Anti-LDL Combined Sorbent to Remove LDL  
 from Plasma; The anti-LDL reagent, as described  
 above, (1.5 mL) was packed into a 1.0 x 10 cm column  
 containing a fritted disc at the bottom to retain the  
 water-insoluble reagent material. The packed reagent  
 was washed with several bed volumes of phosphate  
 buffered saline at pH 7.4 to remove the merthiolate.  
 25     Citrate plasma (9.0 mL) was then recirculated through  
 the column for 1 hr. Samples of the plasma before and  
 after recirculation were assayed to determine the  
 amount of removal of LDL or other constituents from  
 the plasma. The removal of LDL from the plasma is  
 30     illustrated in Figure 1. Quantitation of the  
 lipoprotein levels (HDL and LDL) in plasma is achieved  
 by measuring the cholesterol content of the respective  
 lipoprotein. The values of LDL cholesterol (solid  
 bars) and HDL cholesterol (hatched bars; total  
 35     cholesterol is the sum of HDL and LDL) before and  
 after treatment of the plasma by the anti-LDL  
 water-insoluble reagent are presented. In the studies

29

depicted in Figure 1, two plasma samples were perfused over the reagent. Note that the reagent removes LDL effectively whether a high cholesterol (>300 mg/dl) or a low cholesterol (155 mg/dl) plasma is used. Table VI shows the effect of the plasma recirculation over the water-soluble reagent on the other plasma components before and after recirculation. There were no significant changes in the levels of the other plasma constituents.

15

20

25

30

35

TABLE VI  
SUMMARY OF TESTING FOR CHANGES IN PLASMA CONSTITUENTS

5	TESTS	UNITS	<u>EXPERIMENT 1</u>		<u>EXPERIMENT 2</u>	
			Before	After	Before	After
	LDH	u/l	150.0	137.0	120.0	108.0
	SGOT	u/l	20.0	28.0	26.0	35.0
10	SGPT	u/l	14.0	13.0	30.0	16.0
	BILIRUBIN	mg/dl	0.6	0.3	0.4	0.2
	TOTAL PROTEIN	gm/dl	6.0	5.9	6.1	5.5
	ALBUMIN	gm/dl	3.9	3.7	3.9	3.6
15	GLOBULIN	gm/dl	2.1	2.2	2.2	1.9
	SODIUM	meq/l	155.0	158.0	153.0	144.0
	POTASSIUM	meq/l	3.8	4.5	3.6	3.3
	CHLORIDE	meq/l	84.0	93.0	77.0	87.0
20	PHOSPHOROUS	mg/dl	2.7	4.6	2.1	3.1
	URIC ACID	mg/dl	4.3	3.4	4.2	3.8
	GLUCOSE	mg/dl	83.0	74.0	102.0	93.0
	CREATININE	mg/dl	1.0	1.0	0.8	0.9
	UREA	mg/dl	17.0	16.0	8.0	7.0
25	ALKALINE					
	PHOSPHATASE	u/l	59.0	48.0	68.0	65.0
	TRIGLYCERIDES	mg/dl	111.0	141.0	179.0	137.0
	CHOLESTEROL	mg/dl	155.0	70.0	325.0	175.0
30	HDL	mg/dl	30.0	27.0	46.0	47.0
	LDL	mg/dl	105.0	20.0	248.0	103.0
	ALPHA ANTI-					
	TRYPSIN	mg/dl	170.0	166.0	N/A	178.0



TABLE VI (CONTINUED)  
SUMMARY OF TESTING FOR CHANGES IN PLASMA CONSTITUENTS

5	TESTS	UNITS	<u>EXPERIMENT 3</u>		<u>EXPERIMENT 4</u>	
			Before	After	Before	After
	LDH	u/l	66.0	63.0	45.0	53.0
	SGOT	u/l	13.0	14.0	21.0	4.0
10	SGPT	u/l	17.0	16.0	11.0	6.0
	BILIRUBIN	mg/dl	0.1	0.1	0.2	0.1
	TOTAL PROTEIN	gm/dl	5.2	5.0	5.3	4.9
	ALBUMIN	gm/dl	3.4	3.3	3.4	3.0
15	GLOBULIN	gm/dl	1.8	1.6	1.9	1.9
	SODIUM	meq/l	160.0	160.0	160.0	160.0
	POTASSIUM	meq/l	3.5	3.4	3.3	3.2
	CHLORIDE	meq/l	111.0	113.0	112.0	129.0
20	PHOSPHOROUS	mg/dl	4.9	4.8	4.9	6.8
	URIC ACID	mg/dl	5.0	4.8	5.7	4.8
	GLUCOSE	mg/dl	119.0	115.0	54.0	68.0
	CREATININE	mg/dl	9.7	9.4	10.9	9.7
	UREA	mg/dl	58.0	57.0	59.0	49.0
25	ALKALINE					
	PHOSPHATASE	u/l	45.0	42.0	36.0	30.0
	TRIGLYCERIDES	mg/dl	124.0	105.0	91.0	70.0
	CHOLESTEROL	mg/dl	156.0	102.0	140.0	100.0
30	HDL	mg/dl	24.0	25.0	21.0	20.0
	LDL	mg/dl	132.1	77.0	120.0	80.0
	ALPHA ANTI- TRYPSIN	mg/dl	157.0	150.0	N/A	N/A

32

It should be understood that the detailed description and specific examples provided above, while indicating preferred embodiments of the invention, are given by way of illustration only. From this disclosure one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

15

20

25

30

35

## WHAT IS CLAIMED IS:

1. A water-insoluble reagent for removal of a  
5 substance from physiological fluids comprising (a) a  
carrier substrate, and immobilized on the surface of  
said carrier substrate, (b) a binding ligand specific  
for said substance, and (c) a biocompatibility agent  
10 comprising an organic molecule with a nucleophilic  
moiety and negatively-charged moiety separated by a  
spacer arm, said biocompatibility agent immobilized on  
said surface through reaction with said nucleophilic  
moiety.

2. A water-insoluble reagent of Claim 1 wherein  
15 said carrier substrate is a neutral polymer.

3. A water-insoluble reagent of Claim 2 wherein  
said neutral polymer is selected from the group  
consisting of Affigel 10, Affigel 15,  
Tresyl-Sepharose, CNBr-Sepharose, Reacti-Gel (CDI  
20 activated agarose/dextran), Eupergit C,  
epoxy-activated Sepharose, epoxy-activated silica,  
carboxy Sepharose, Thiol(activated) Sepharose,  
trityl-agarose, oxirane acrylic beads, p-nitrophenol  
agarose, tosyl-agarose, periodate-treated agarose,  
25 activated CM-cellulose, activated cellulose,  
poly-glycidyl methacrylate, PEI-glutaraldehyde  
activated nylon, PEI-glutaraldehyde activated silica,  
glutaraldehyde-activated agarose, isocyanate-activated  
agarose, maleimide-activated agarose, and  
30 chloromethylpolystyrene.

4. A water-insoluble reagent of Claim 3 wherein  
said neutral polymer is tresyl-Sepharose.

5. A water-insoluble reagent of Claim 1 wherein  
said binding ligand is selected from the group  
35 consisting of a monoclonal antibody, a polyclonal  
antibody, hormone-receptor, lectin, antigen and  
vitamin binding protein.

6. A water-insoluble reagent of Claim 5 wherein said binding ligand is a monoclonal antibody specific for the substance to be removed from a physiological fluid.

7. A water-insoluble reagent of Claim 6 wherein said monoclonal antibody is specific for low density lipoprotein.

8. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent comprises an organic molecule with said negatively-charged moiety selected from the group consisting of  $\text{OSO}_3^-$ ,  $\text{RSO}_3^-$ ,  $\text{RSO}_2^-$ , and  $\text{COO}^-$ .

9. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent comprises an organic molecule with said nucleophilic moiety selected from the group consisting of  $\text{NH}_2$ ,  $\text{SH}$ , and  $\text{OH}$ .

10. A water-insoluble reagent of Claim 1 wherein said biocompatibility agent is selected from the group consisting of sulfamic acid, taurine, sulfanilic acid, glycine, aspartate, cysteine, isethionic acid, Hydroxybenzenesulfonic Acid, glucose sulfate, Lactic Acid, glucuronic acid, gluconic acid, 2-mercaptoethanesulfonic Acid, Mercaptoacetic Acid, Thiosalicylic Acid, Thiolactic Acid.

11. A water-insoluble reagent of Claim 10 wherein said biocompatibility agent is sulfamic acid.

12. A process for preparing a water-insoluble reagent for removal of a substance from physiological fluids comprising the step of contacting and reacting a carrier substrate with an aqueous solution of a binding ligand specific for said substance and a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm.

35

13. The process of Claim 12 wherein the concentration of said binding ligand is from about 10 mg/ml to about 100 mg/ml and the concentration of said biocompatibility agent is from about 0.01 M to about 5.0M.

14. The process of Claim 13 wherein the pH is controlled with a non-nucleophilic buffer in the range of from about 4 to about 10.

15. A process for preparing a water-insoluble reagent for removal of a substance from physiological fluids comprising the steps: (a) contacting and reacting a carrier substrate with an aqueous solution of binding ligand specific for said substance, and (b) contacting and reacting the product of step (a) with an aqueous solution of a biocompatibility agent comprising an organic molecule with a nucleophilic moiety and a negatively-charged moiety separated by a spacer arm to produce said water-insoluble reagent.

16. The process of Claim 15 wherein the concentration of said binding ligand is from about 10 mg/ml to about 100 mg/ml and the concentration of said biocompatibility agent is from about 0.01M to about 5.0M.

17. The process of Claim 15 wherein the pH is controlled with a non-nucleophilic buffer in the range of from about 4 to about 10.

18. A method of treating a physiological fluid to remove a substance comprising contacting said fluid with a water-insoluble reagent of Claim 1.

19. The method of Claim 18 wherein said physiological fluid is mammalian plasma.

20. The method of Claim 18 wherein said substance is low density lipoproteins.

21. An apparatus for the extracorporeal treatment of physiological fluids to remove a specific substance comprising means for withdrawing whole

36

blood from a mammal, means for separating plasma from  
the whole blood, means for treating said plasma  
including a chamber containing a water-insoluble  
5 reagent of Claim 1 which will interact with and  
deplete said substance from the plasma, and means for  
recombining the substantially substance depleted  
plasma with the remainder of the whole blood and for  
returning the recombined whole blood to the mammal.

10 22. The apparatus of Claim 21 wherein the water  
insoluble reagent comprises Tresyl-Sepharose having  
monoclonal antibody for low density lipoprotein and  
sulfamic acid immobilized on the surface of said  
Tresyl-Sepharose.

15

20

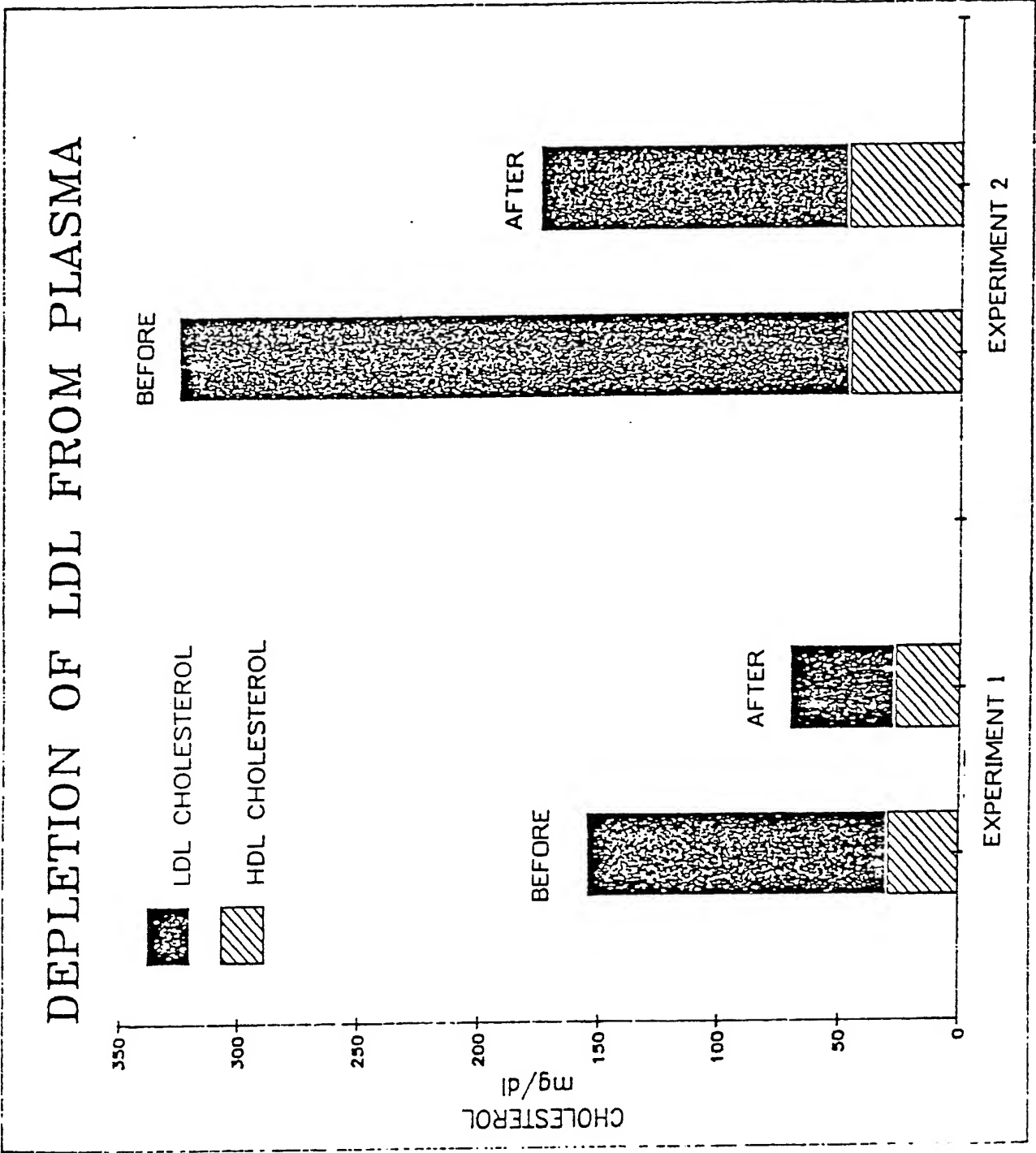
25

30

35

1/1

FIGURE 1



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02067

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC INT CL. (4): A01N 1/02; A61M 37/00; E01D 15/00; G01N 33/543 US. CL.: 210/679; 427/2; 436/518,527,530,531,824,825; 530/413; 604/5,6		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S. CL	210/434,679; 427/2; 436/518,527,529, 530, 531, 824, 825; 530/413; 604/5,6	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
APS SEARCH: S Sulfamic and (Antigagula? or Extracorporeal or plasmapheresis)		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	US, A, 4,627,915 (KURODA ET AL.) 9 December 1986. See col. 4, lines 52-65; col. 7, lines 7-13; col. 7, line 64 - col. 8, line 25; col. 9, line 51 - col. 10, line 37; col. 16, lines 12-20.	1-3, 8-19,21
A	US, A, 4,693,985 (DEGEN ET AL.) 15 September 1987. See col. 3, lines 22-54.	18,19
A	US, A, 4,737,544 (MCCAIN ET AL.) 12 April 1988. See summary.	21
Y	JA, A, 59-0,189,859 (ASHI CHEMICAL IND. KK) 27 October 1984. See Derwent Abstract.	1-21
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search 15 September 1988		Date of Mailing of this International Search Report 9 NOV 1988
International Searching Authority ISA/US		Signature of Authorized Officer <i>David A. Saunders</i> DAVID A. SAUNDERS



## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
<u>X</u> Y	JA, A 60-0,087,854 (ASHI-CHEMICAL IND. KK) 17 May 1985. See entire document.	1-3,5, 8-21 4,6,7, 22
Y	JA, A, 60-0,239,425 (TEIJIN KK) 28 November 1985. See Derwent Abstract	6,7,20, 22
Y	Biochimica ET Biophysica Acta, Vol. 876, No., Issued 21 March 1986, (Amsterdam, NL) KOREN ET AL., "Characterization of a monoclonal antibody that binds equally to all apolipoprotein B. II. isolation of apolipoprotein and lipoprotein forms of human plasma apolipoprotein B. containing lipoproteins from human plasma", pp. 101-107. See abstract.	6,7,20, 22
Y	Biochem. Biophys. Research Commun., Vol. 102, No. 1, issued 16 September 1981 (New York, NY, USA) NILSSON ET AL, "Immobilization of enzymes and affinity ligands to various hydroxyl group carrying supports using highly reactive sulfonyl chlorides" pp. 449-457. See pp. 449-450.	4,22
Y	Proc. Natl. Acad. Sci. USA, Vol. 78, No. 1., issued January 1981 (Washington, D.C. USA) STOFFEL ET AL., Selective Removal of apolipoprotein B-containing serum lipoproteins from blood plasma". pp 611-615. See abstract.	6,7,20, 22
Y	Proc. Natl. Acad. Sci. USA, Vol. 83, No. 2, issued February 1986 (Washington D.C., USA) PARKER ET AL., "Plasma high density lipoprotein is increased in man when low density lipoprotein (LDL) is lowered by LDL-pheresis". pp. 777-781. See abstract.	20,22